FGFR3^{S249C} mutation promotes chemoresistance by activating Akt signaling in bladder cancer cells

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Abstract. Fibroblast growth factor receptor 3 (FGFR3) is a high frequency mutant gene in bladder cancer (BCa) and has become a promising therapeutic target due to its involvement in cell proliferation and migration. However, whether and how FGFR3 mutations affects BCa cell chemosensitivity is unknown. The current study aimed to elucidate the role of the FGFR3^{S249C} mutation in the development of chemoresistance in BCa cells. The results revealed that 97-7 (FGFR3^{S249C}) cells had decreased sensitivity to cisplatin compared with 5637 (FGFR3^{WT}) and T24 (FGFR3^{WT}) cells. The ratio of phosphorylated-Akt/total-Akt was higher in 97-7 (FGFR3^{S249C}) cells, which was reversed by knockdown of FGFR3. Furthermore, inhibition of Akt signaling by GDC0068 or LY294002 increased the cisplatin sensitivity of 97-7 (FGFR3^{S249C}) cells. GDC0068 or LY294002 was also revealed to augment the effects of cisplatin on 97-7 (FGFR3^{S249C}) cell proliferation and apoptosis. The results of the present study demonstrated that the FGFR3^{S249C} mutation promotes chemoresistance in BCa cells by activating the Akt signaling pathway. The FGFR3^{S249C} mutation may therefore be used as a predictor of chemosensitivity in patients with BCa.

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Introduction

Bladder cancer (BCa) is the second most common malignancy of the genitourinary tract (1), with ~76,960 new diagnoses and 16,390 BCa associated deaths reported in the USA in 2016 (2). Since 1984, the number of BCa diagnoses has increased by~36% per year (2). Urothelial carcinoma (transitional cell carcinoma) is the most common BCa (>90%) and is generally classified as non-muscle invasive BCa (NMIBC) or muscle invasive BCa (MIBC), according to the nature of the tumor (3). Among NMIBC cases, 50-70% recur after treatment and an estimated 10-15% develop into MIBC, which possesses the characteristics of metastatic malignant tumors, exhibiting a 5-year survival rate of 50-60% (4,5). When MIBC progresses to metastatic BCa, the 5-year survival rate decreases to 5% (4). Cisplatin-based combination chemotherapy is widely used in MIBC treatment and increases survival (6). However, the response rate to chemotherapy is only ~50% and resistance to cisplatin is considered to be the principal cause of this poor response (7). An in-depth study of possible factors affecting cisplatin sensitivity is therefore required to identify potential novel therapeutic targets for BCa and subsequently improve clinical treatment outcomes.

Fibroblast growth factor receptor 3 (FGFR3), one of the four highly conserved FGFRs, is a receptor tyrosine kinase involved in cancer cell proliferation and migration (8). A total of 11 different FGFR3-activating missense mutations have been reported in ~70% of NMIBC and 15% of MIBC cases (8,9). An analysis of the latest mutation data of BCa tissue from The Cancer Genome Atlas database (http://www.cbioportal. org/index.do) revealed that 14% of patients with BCa exhibited FGFR3-activating point mutations (10,11). Among the FGFR3 mutations, S249C accounted for 53.4% and was the most frequent mutation in BCa (10,11). S249C, located on exon 7, induces a substitution of serine with cysteine at codon 249. This substitution leads to a ligand-independent dimerization and the auto-phosphorylation of FGFR3, which results in the continued activation of downstream proliferative pathways (12). A study has demonstrated that FGFR3 may be an oncogenic driver of BCa and hence targeting FGFR3 may be a beneficial therapeutic approach (13). However, whether

FGFR3 mutations, particularly the major mutation S249C, are involved in chemoresistance of BCa remains unknown.

The current study aimed to elucidate the function and underlying mechanism of the FGFR3^{S249C} mutation in the development of chemoresistance in BCa cells, to identify potential therapeutic targets for patients with BCa and for those who carry this mutation.

Materials and methods

Cell culture. The BCa cell line 97-7 expressing endogenous mutant FGFR3^{S249C} (12,14) was provided by Professor Margaret Knowles at the Leeds Institute of Cancer and Pathology, Cancer Research UK Clinical Centre, St. James's University Hospital (Leeds, UK). The FGFR3 wild-type (FGFR3^{WT}) BCa cell line 5637 and T24 were obtained from the Cell Bank of the Chinese Academy of Sciences. 97-7 (FGFR3^{S249C}) cells were cultured in Hams F12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% (v/v) FBS (Gibco; Thermo Fisher Scientific, Inc.), 1x Insulin-Transferrin-Selenium (Sigma-Aldrich; Merck KGaA), 1x non-essential amino acids (Gibco; Thermo Fisher Scientific, Inc.) and 1 μg/ml hydrocortisone (Sigma-Aldrich; Merck KGaA). 5637 (FGFR3^{WT}) and T24 (FGFR3^{WT}) cells were cultured in RPMI 1640 and McCoy's 5A medium (all Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% (v/v) FBS. All cell lines were cultured in a humidified incubator at 37˚C with 5% CO_2.

Chemosensitivity assay. 5637 (FGFR3^{WT}), T24 (FGFR3^{WT}) and 97-7 (FGFR3^{S249C}) cells were seeded in 96-well plates at a density of 6,000 cells/well in RPMI 1640 medium, McCoy's 5A medium and Hams F12 medium, respectively, containing 10% FBS. Following overnight attachment in a humidified incubator at 37˚C with 5% CO_2, the medium was discarded and 5637 (FGFR3^{WT}) and T24 (FGFR3^{WT}) cells were incubated at 37˚C with various concentrations of cisplatin (0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40 and 80 μg/ml; MedChemExpress LLC) for 48 h. For the 97-7 (FGFR3^{S249C}) cells, medium was discarded following overnight attachment at 37˚C and cells were treated with different concentrations of cisplatin (0, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 100, 200 and 400 μg/ml with or without the pan-Akt inhibitor GDC-0068 (10 μM; MedChemExpress LLC), or a broad-spectrum PI3K inhibitor LY294002 (20 μM; MedChemExpress LLC) were used for 48 h. After incubation at 37˚C, cell viability was detected in all cells using a Cell Counting kit-8 (CCK-8) assay kit (Dojindo Molecular Technologies, Inc.) in accordance with the manufacturer's protocol. Optical density (OD) values were recorded using a microplate reader (Bio-Rad Laboratories, Inc.) at 450 nm. The ratio of cisplatin inhibition, GDC-0068, LY294002 or a combination treatment at each concentration was calculated as follows: (the OD value of experimental groups - the OD value of blank groups)/(the OD value of control groups - the OD value of blank groups). Concentration-viability curves were presented using Graph Pad Prism 7.0 software (GraphPad Software, Inc.). Half maximal inhibitory concentration (IC_{50}) values were subsequently determined.

Western blot analysis. 5637 (FGFR3^{WT}), T24 (FGFR3^{WT}) and 97-7 (FGFR3^{S249C}) cells were lysed in cell lysis buffer (Sigma-Aldrich; Merck KGaA) supplemented with protease inhibitors (Roche Diagnostics). Protein concentration was determined with a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Inc.). Protein (30 μg) underwent 12% SDS-PAGE and was transferred to PVDF membranes (EMD Millipore). After blocking with 5% skimmed milk at room temperature for 1 h membranes were overnight incubated overnight at 4˚C with primary antibodies against phosphorylated-Akt (P-Akt; 1:600; cat. no. 9271; Cell Signaling Technology, Inc.), total-Akt (T-Akt; 1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.), FGFR3 (1:500; cat. no. AP14841c-400; Abgent Biotech Co., Ltd.) and β-actin (1:2,500; cat. no. A5441; Sigma-Aldrich; Merck KGaA). Secondary anti-mouse IgG (H+L), HRP conjugated (1:5,000; cat. no. SA00001-1; Proteintech Group, Inc.) or anti-rabbit IgG (H+L), HRP conjugated (1:5,000; cat. no. SA00001-2; Proteintech Group, Inc.) antibodies were incubated at room temperature for 2 h and blots were developed using the SuperSignal™ West Dura Extended Duration Substrate reagent (Thermo Fisher Scientific, Inc.) with an Amersham Imager 600 (GE Healthcare). Band intensity was determined by ImageJ 2X software (National Institutes of Health) and normalized to β-actin. The activation of the Akt signaling pathway was determined using the ratio of P-Akt/T-Akt.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from 97-7 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse-transcribed using a PrimeScript RT reagent kit with a gDNA Eraser (Takara Bio, Inc.) for cDNA synthesis and genomic DNA removal. RT-qPCR was performed using a QuantiNova™ SYBR Green PCR mix kit (Qiagen GmbH) and performed using Applied Biosystems Prism 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification was performed according to the reaction conditions of 95˚C for 10 min; 95˚C for 10 sec, 60˚C for 30 sec, for 40 cycles. The relative expression of FGFR3 was compared with that of β-actin and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method (15). The primers used were as follows: FGFR3 forward, 5'-TGCGTCGTGGAGAACAAG TTT-3' and reverse, 5'-GCACGGTAACGTAGGGTGTG-3'; β-actin forward, 5'-CTGGAACGGTGAAGGTGACA-3' and reverse, 5'-AAGGGACTTCCTGTAACAATGCA-3'.

RNA interference assay. RNA interference was used to knockdown the expression of FGFR3 in 97-7 (FGFR3^{S249C}) cells. Small interfering RNA (siRNA) for FGFR3 (siRNA-FGFR3) and scrambled negative control (NC) siRNA (siRNA-NC) were obtained from Shanghai GenePharma Co., Ltd. 97-7 (FGFR3^{S249C}) cells were transfected with 30 nM siRNA using lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in six-well plates. After 48 h, cells were collected and used for RNA and protein extraction. The siRNA sequences were as follows: siRNA-NC forward, 5'-UUCUCC GAACGUGUCACGUTT-3' and reverse, ACGUGACACGUU CGGAGAATT; siRNA-FGFR3 forward, 5'-GCUGAAAGA CGAUGCCACUTT-3' and reverse, 5'-AGUGGCAUCGUC UUUCAGCTT-3'.

Cell proliferation assay. A CCK-8 assay was performed to determine cell proliferation as described in a previous

study (16). 97-7 ($FGFR3^{S249C}$) cells were seeded into 96-well plates at a density of 5,000 cells per well and incubated at 37˚C with 5 μg/ml cisplatin with or without 10 μM GDC0068 and 10 μg/ml cisplatin with or without 20 μM LY294002. These concentrations were selected based on the IC_{50} values of treatments. Following incubation for 1, 2, 3, and 4 days, the cell morphology was assessed using a light microscope (Nikon Corporation), and cell proliferation was detected using a CCK-8 assay kit (Dojindo Molecular Technologies, Inc.) in accordance with the manufacturer's protocol. OD values at 450 nm were measured using a microplate reader.

Cell apoptosis analysis. Cell apoptosis analysis was performed using an Annexin V-FITC/propidium Iodide (PI) Cell Apoptosis Analysis kit according to manufacturer's protocol (Absin Bioscience, Inc.). Cells were treated with 5 μg/ml cisplatin with or without 10 μM GDC0068 and 10 μg/ml cisplatin with or without 20 μM LY294002 at 37˚C for 72 h. 97-7 ($FGFR3^{S249C}$) cells were harvested and washed twice in ice-cold PBS, resuspended in 300 μl binding buffer (Absin Bioscience, Inc.) and incubated with 5 μl Annexin V-FITC and 10 μl PI in the dark for 15 min at room temperature. After adding 200 μl PBS to each sample, cell apoptosis was detected within 1 h using a flow cytometer (Beckman Coulter, Inc.). The results were analysed using FlowJo v10 software (FlowJo LLC).

Statistical analysis. Analyses were performed using SPSS 23.0 software (IBM Corp.). Statistical analysis was performed using a two-tailed Student's t-test or one-way ANOVA, followed by a Newman-Keuls or a Dunnett's multiple comparison test. All data were presented as the mean ± SEM. $P<0.05$ was considered to indicate a statistically significant difference.

Results

97-7 ($FGFR3^{S249C}$) cells are more resistant to cisplatin treatment compared with 5637 ($FGFR3^{WT}$) and T24 ($FGFR3^{WT}$) cells. Cell viability was assessed using a CCK-8 assay after treatment with different concentrations of cisplatin for 48 h, to determine whether cisplatin sensitivity was significantly different between the BCa cells carrying the S249C mutant FGFR3 (97-7) and wild type FGFR3 (5637 and T24). The results revealed that cell viability was reduced following cisplatin treatment in all three cell lines (Fig. 1A, C and E). Concentration-viability curves revealed that the IC_{50} of cisplatin was ~21 and ~28 fold higher in 97-7 cells ($FGFR3^{S249C}$), compared with 5637 ($FGFR3^{WT}$) and T24 ($FGFR3^{WT}$) cells (44.63 μg/ml vs. 2.07 μg/ml and 1.58 μg/ml, respectively; Fig. 1B, D and F). 97-7 ($FGFR3^{S249C}$) cells were therefore more resistant to cisplatin treatment compared with 5637 ($FGFR3^{WT}$) and T24 ($FGFR3^{WT}$) cells.

Akt signaling pathway is activated in 97-7 ($FGFR3^{S249C}$) cells. Previous studies have demonstrated that the activation of Akt signaling serves an important role in cancer cell chemoresistance (17,18). It has also been revealed that the phosphorylation of FGFR3 leads to the activation of several downstream signaling cascades, including PI3K-Akt-mTOR and Ras-Raf-mitogen activated protein kinase (MAPK) (13,19). These data revealed that $FGFR3^{S249C}$ mutations in BCa cells may induce chemoresistance by activating Akt signaling. Therefore, levels of T-Akt and P-Akt were detected in the present study using western blot analysis in 97-7 ($FGFR3^{S249C}$), 5637 ($FGFR3^{WT}$) and T24 ($FGFR3^{WT}$) cells. The activation of the Akt signaling pathway was determined using the ratio of P-Akt/T-Akt. As presented in Fig. 2A-C, 97-7 ($FGFR3^{S249C}$) cells exhibited increased P-Akt levels compared with 5637 ($FGFR3^{WT}$) and T24 ($FGFR3^{WT}$) cells, although no marked change in T-Akt levels were observed (Fig. 2A). The elevated P-Akt/T-Akt ratio in 97-7($FGFR3^{S249C}$) cells indicated the activation of Akt signaling (Fig. 2B).

To further verify the regulatory effect of FGFR3 on Akt signaling, the P-Akt/T-Akt ratio was assessed in 97-7 ($FGFR3^{S249C}$) cells after FGFR3 knockdown using siRNA for 48 h. Consistent with a previous study (19), the knockdown of FGFR3, which decreased FGFR3 mRNA and protein expression levels (Fig. 2C and D), resulted in a ~30% decrease in the ratio of P-Akt/T-Akt (Fig. 2E and F). The results indicated that the $FGFR3^{S249C}$ mutation, which leads to the auto-phosphorylation of FGFR3 in 97-7 ($FGFR3^{S249C}$) cells, augments Akt signaling.

Inhibition of the Akt signaling pathway sensitizes 97-7 ($FGFR3^{S249C}$) cells to cisplatin. To determine the role of Akt signaling in cisplatin resistance, 97-7 ($FGFR3^{S249C}$) cells treated with cisplatin were subsequently treated with or without the Akt inhibitor (GDC0068) or the PI3K inhibitor (LY294002) for 48 h, following which cell viability was assessed. GDC0068 <16 μM and LY294002 <80 μM did not affect cell viability in 97-7 ($FGFR3^{S249C}$) cells (Fig. 3A and B). Western blot analysis revealed that 20 μM LY294002 decreased the level of P-Akt and inhibited the activation of Akt signaling (Fig. 3C). GDC0068, is an ATP-competitive Akt inhibitor (20), which occupies the ATP binding pocket of Akt kinases and facilitates intramolecular interactions of phosphorylated T308/S473 with two residues in the catalytic cleft (R273, H194), results in restricting phosphatase access and sustaining Akt phosphorylation (21). Therefore, as other previous studies (20,22,23), although GDC0068 inhibits the activity of Akt signaling, a high level of P-Akt can be detected after GDC0068 treatment (Fig. 3C). Based on these data, 10 μM GDC0068 and 20 μM LY294002 were utilized in subsequent combined treatment studies. GDC0068 and LY294002 increased the sensitivity of 97-7 ($FGFR3^{S249C}$) cells to cisplatin (Fig. D and F). The IC_{50} value was reduced from 44.11 μg/ml for treatment with cisplatin alone to 4.17 and 8.91 μg/ml when administered in combination with GDC0068 and LY294002, respectively (Fig. 3E and G). The results revealed that the Akt signaling pathway may be involved in the chemoresistance of 97-7 ($FGFR3^{S249C}$) cells.

Inhibition of the Akt signaling pathway augments the effects of cisplatin on 97-7 ($FGFR3^{S249C}$) cell proliferation and apoptosis. To assess the potential role of the Akt signaling pathway in cisplatin-induced apoptosis, the effect of Akt inhibitors on the proliferation and apoptosis of 97-7 ($FGFR3^{S249C}$) cells was determined following treatment with cisplatin. The results revealed that cisplatin treatment altered the cell from fusiform to round, and reduced cell numbers, which were more pronounced after combinational treatment

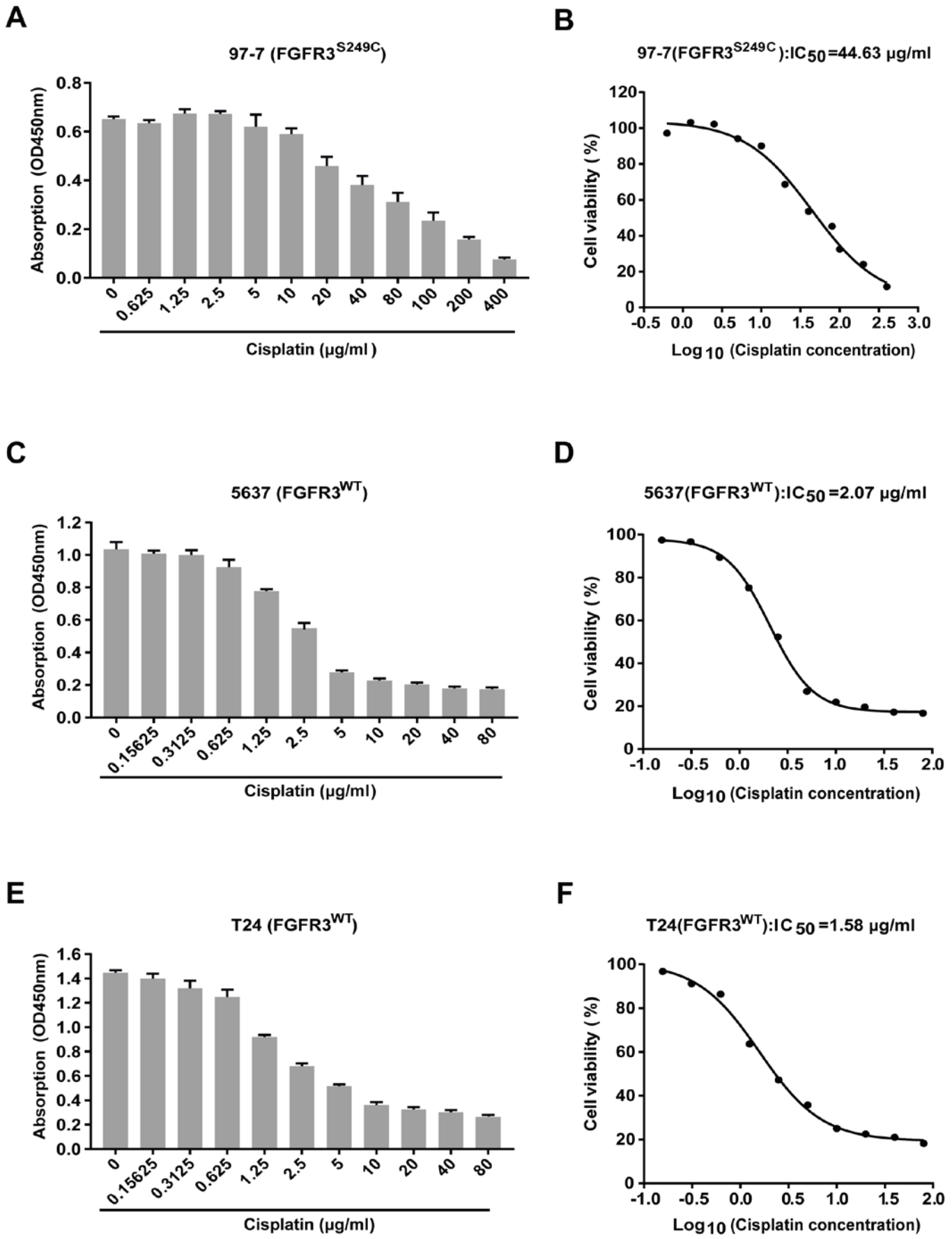

Figure 1. 97-7 (FGFR3^{S249C}) cells revealed higher resistance to cisplatin treatment. (A) CCK-8 analysis of 97-7 (FGFR3^{S249C}) cell viability and (B) its respective concentration-viability curve; (C) CCK-8 analysis of 5637 (FGFR3^{WT}) cell viability and (D) its respective concentration-viability curve; (E) CCK-8 analysis of 97-7 (FGFR3^{S249C}) cell viability and (F) its respective concentration-viability curve 48 h after cisplatin treatment at different concentrations. FGFR3, fibroblast growth factor receptor 3; CCK-8, cell counting kit-8; WT, wild-type; OD, optical density; IC_{50}, half maximal inhibitory concentration.

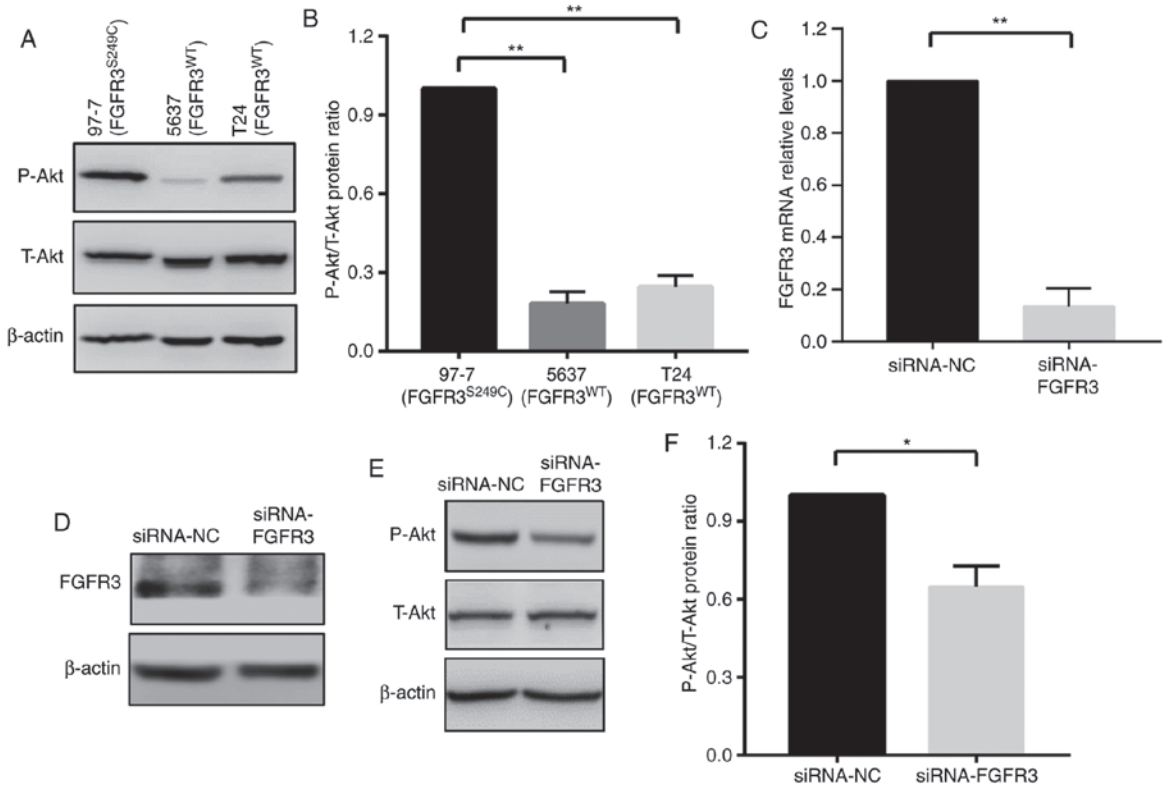

Figure 2. Akt signaling pathway was activated in 97-7 (FGFR3^{S249C}) cells. (A) Western blot analysis of protein expression and (B) quantification of results in 97-7 (FGFR3^{S249C}), 5637 (FGFR3^{WT}) and T24 (FGFR3^{WT}) cells. (C) Reverse transcription-quantitative PCR analysis of FGFR3 mRNA expression in 97-7 (FGFR3^{S249C}) cells after transfection with siRNA-FGFR3 for 48 h. (D) Western blot analysis of 97-7 (FGFR3^{S249C}) cells after transfection with siRNA-FGFR3 or siRNA-NC as a control for 48 h, with (E) P-Akt and T-Akt ratio and (F) quantification via densitometry. β-actin was used as an internal control for western blot analysis. Data are presented as the mean ± SEM from 3 independent experiments. *P<0.05; **P<0.01. FGFR3, fibroblast growth factor receptor 3; siRNA, small interfering RNA; NC, negative control; P-Akt, phosphorylated Akt; T-Akt, total-Akt.

with GDC0068 or LY294002 (Fig. 4A and B). The suppression of cell proliferation by cisplatin was more apparent with prolonged treatments and the combined treatment of cisplatin with GDC0068 or LY294002 significantly increased the effect of cisplatin on the proliferation of 97-7 cells (FGFR3^{S249C}; Fig. 4C and D). After 72 h of treatment, 5 and 10 μg/ml cisplatin significantly increased apoptosis compared with untreated cells (Fig. 4E and F). Furthermore, GDC0068 and LY294002 in combination with cisplatin significantly increased apoptosis by 53.3% and 25%, respectively, compared with cisplatin treatment alone (the numbers of apoptotic cells were 23% vs. 15% and 25% vs. 20%, respectively; Fig. 4E and F). The results of the current study indicate that the Akt signaling pathway is activated in FGFR3^{S249C} mutant BCa cells, resulting in the promotion of cisplatin resistance, and the chemical inhibition of the Akt signaling pathway in 97-7 (FGFR3^{S249C}) cells indicated improved chemosensitivity (Fig. 5).

Discussion

BCa is the most common malignant carcinoma of the urinary system (1). Despite initial sensitivity to standard first-line combination cisplatin-based chemotherapy, the overall prognosis of MIBC is poor (8,24). Since chemoresistance is a critical factor that effects clinical treatment outcomes (7), studies that assess and develop efficient chemosensitizers have become an urgent requirement. FGFR3, a high-frequency mutant gene in BCa with known regulatory roles in tumor progression, has become a promising therapeutic target (8,25,26). In the present study, the role of S249C mutant FGFR3 was elucidated in the development of BCa cell chemoresistance. The results revealed that BCa cells carrying FGFR3^{S249C} exhibited a lower sensitivity to cisplatin. The FGFR3^{S249C} mutation in BCa cells also activated Akt signaling and the chemical inhibition of the Akt signaling pathway in 97-7 (FGFR3^{S249C}) cells improved chemosensitivity and enhanced the cisplatin-induced suppression of cell proliferation and apoptosis induction.

Conventional chemotherapeutic drugs, including platinum and fluorouracil induce an anti-tumor effect by damaging DNA and promoting tumor cell apoptosis (27). As cisplatin-based combination chemotherapy is the most important therapeutic strategy for MIBC (6), cisplatin resistance in BCa cells was assessed in the current study. S249C accounts for >50% of all FGFR3 point mutations in BCa (10,11) and therefore the effect of FGFR3^{S249C} on BCa cell chemosensitivity was also assessed in the present study. The cell line 97-7 was utilized as it carried the FGFR3^{S249C} mutation without additional mutations in Ras, Akt or PIK3CA (Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) (12,14). The 97-7 cell line was established from a primary tumor with invasive transitional cell carcinoma (Grade II/III Stage I) and was demonstrated to express similar FGFR3 levels as low-stage and low-grade tumors (28). In the present study, it was demonstrated that 97-7 cells carrying S249C mutant FGFR3 exhibited a higher IC_{50}

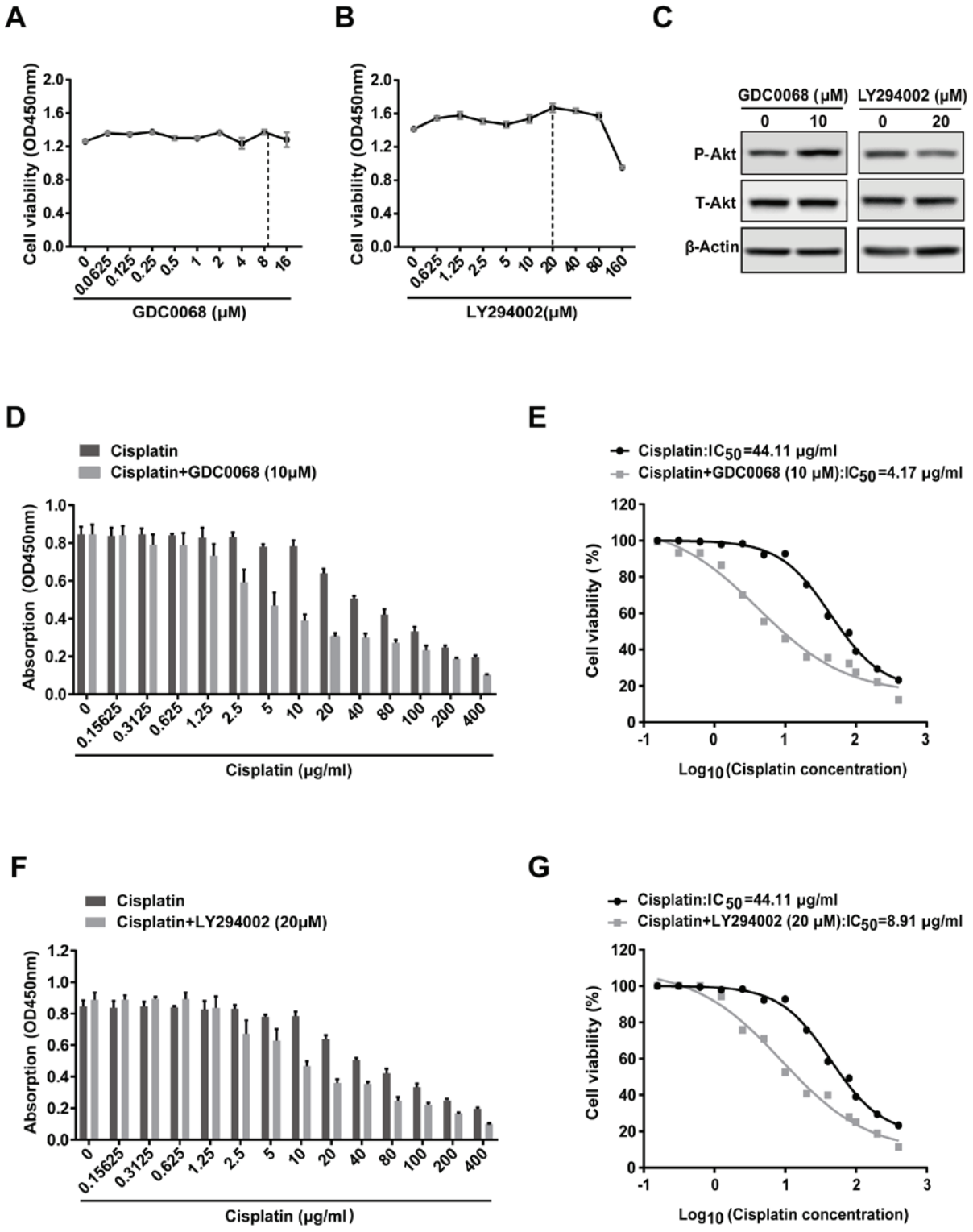

Figure 3. Inhibition of Akt signaling pathway sensitized 97-7 (FGFR3^{S249C}) cells to cisplatin. CCK-8 assay analysis of cell viability after 97-7 (FGFR3^{S249C}) cells were treated with different concentrations of (A) GDC0068 or (B) LY294002 for 48 h. (C) Western blot analysis of T-Akt and P-Akt protein levels after 97-7 (FGFR3^{S249C}) cell treatment with 10 μM GDC0068 or 20 μM LY294002 for 48 h. (D) Absorption of 97-7 (FGFR3^{S249C}) cells treated with cisplatin and cisplatin in combination with 10 μM GDC0068 with its respective (E) concentration-viability curve. (F) Absorption of 97-7 (FGFR3^{S249C}) cells treated with cisplatin and cisplatin in combination with 20 μM LY294002 and (G) its respective concentration-viability curve. The dotted line represents the concentrations of GDC0068 and LY294002 which were used in the subsequent experiments. FGFR3, fibroblast growth factor receptor 3; CCK-8, cell counting kit-8; T-Akt, total-Akt; P-Akt, phosphorylated Akt; OD, optical density; IC_{50}, half maximal inhibitory concentration.

value for cisplatin, indicating that the FGFR3^{S249C} mutation reduced BCa cell sensitivity to cisplatin. This result indicated that the FGFR3^{S249C} mutation may be developed as a predictor of chemosensitivity in patients with BCa.

The mechanisms underlying the development of chemoresistance are complex and include increased drug efflux, alterations in chemotherapy drug targets and the abnormal activation of key signaling pathways such as PI3K/Akt and

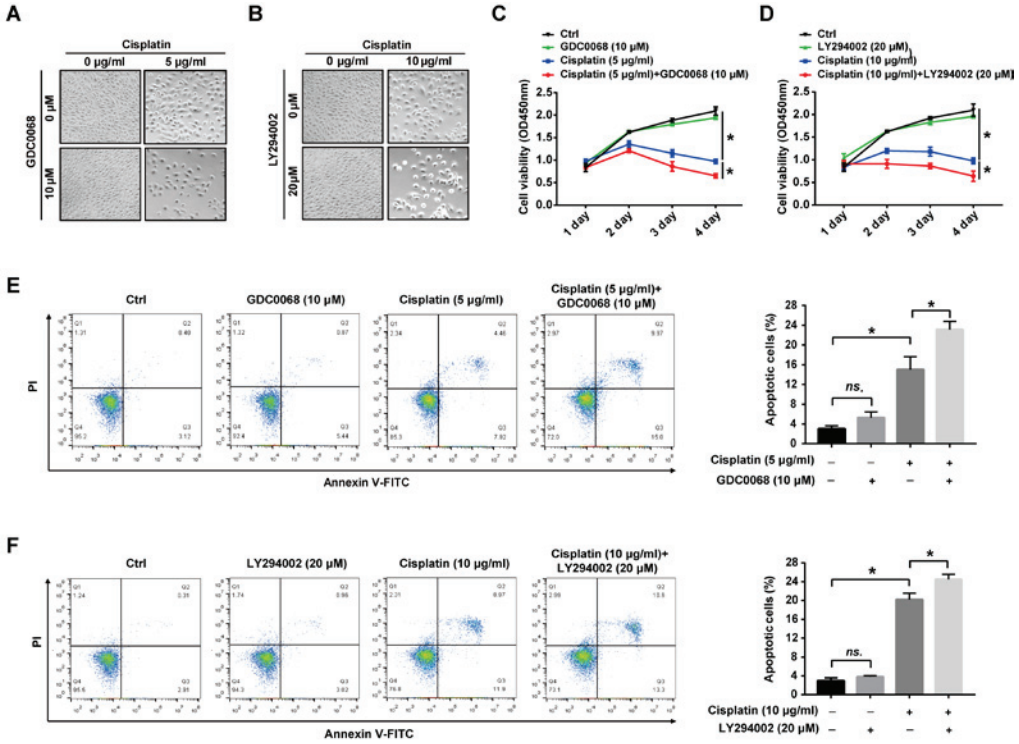

Figure 4. Inhibition of Akt signaling augmented the effects of cisplatin on 97-7 (FGFR3^{S249C}) proliferation and apoptosis. 97-7 (FGFR3^{S249C}) cells were treated with (A) 5 μg/ml cisplatin, 10 μM GDC0068 or these combined treatments and (B) 10 μg/ml cisplatin, 20 μM LY294002 or these combined and morphology was assessed by light microscope (magnification x200) after treatment for 72 h. (C and D) Cell proliferation was subsequently measured via cell counting kit-8 analysis for each aforementioned treatment at the indicated times. (E) Annexin V-FITC/PI staining and flow cytometry analysis of apoptotic cells was performed for (E) GDC0068 and (F) LY294002 treatment combinations after 72 h. Data are presented as the mean ± SEM from 3 independent experiments. *P<0.05. FGFR3, fibroblast growth factor receptor 3; OD, optical density; NS, not significant.

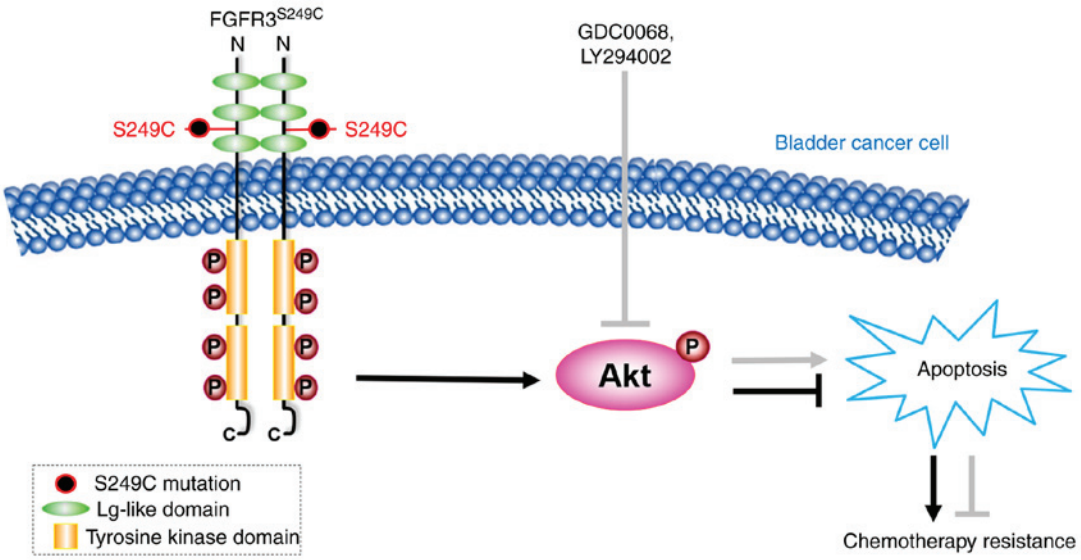

Figure 5. Schematic summary of FGFR3^{S249C} mutation promotes chemotherapy resistance in bladder cancer cells. FGFR3, fibroblast growth factor receptor 3. P, phosphorylation; N, amino terminal; C, carboxyl terminal.

signal transducer and activator of transcription 3 (17,18). Evidence from previous studies has demonstrated that FGFR3 phosphorylation activates several signaling pathways, including the PI3K-Akt-mTOR and Ras-Raf-MAPK pathway (13,19). The Akt signaling pathway may therefore be involved in the FGFR3^{S249C} mutation-induced chemoresistance of BCa cells. In the current study, the ratio of P-Akt/T-Akt increased in 97-7 (FGFR3^{S249C}) cells compared with 5637 (FGFR3^{WT}) and T24 (FGFR3^{WT}; Fig. 2A and B). Furthermore, as reported previously (19), the P-Akt/T-Akt ratio in 97-7 (FGFR3^{S249C}) cells was reversed by FGFR3 knockdown. The results demonstrated that the FGFR3^{S249C} mutation in BCa cells may activate the Akt signaling pathway.

Previous studies have reported that high concentrations of Akt inhibitor (GDC0068) and PI3K inhibitor (LY294002) promotes tumor cell apoptosis and inhibits proliferation (29) (30,31). To avoid these effects, the current study utilized low concentrations of GDC0068 (10 μM) and LY294002 (20 μM). Consistent with previous studies (30,32), 20 μM LY294002 inhibited the Akt signaling pathway and reduced P-Akt levels. GDC0068, is an ATP-competitive Akt inhibitor that occupies the ATP binding pocket of Akt kinases and facilitates intramolecular interactions, resulting restricts phosphatase access and sustains Akt phosphorylation (15,21). This may explain the high P-Akt level detected after treatment with 10 μM GDC0068 in the current study and other previous studies (20,22,23). The inhibition of Akt signaling with GDC0068 or LY294002 sharply decreased the IC_{50} of cisplatin and improved the chemosensitivity of BCa cells carrying S249C mutant FGFR3, enhancing the cisplatin-mediated reduction of proliferation and the induction of apoptosis. The results verified the hypothesis that the Akt signaling pathway may be involved in the FGFR3^{S249C} mutation-induced chemoresistance of BCa cells.

Currently, several Akt inhibitors including GDC0068, MK2206, GSK2110183 and AZD5363 have entered phase II of clinical trials, but the efficacy of therapies with Akt inhibitors as single agents is limited (33). A previous study therefore combine Akt inhibitors with other compounds to increase efficacy (30). For example, a recent study combining cisplatin with MK2206 showed synergistic activity *in vivo* and *in vitro* in lung cancer (34). Furthermore, MK2206 also increased the sensitivity to paclitaxel and carboplatin in melanoma cell lines (35). The results of the current study demonstrated that the use of Akt inhibitors combined with cisplatin-based chemotherapy may be used as a potential treatment strategy for patients with BCa carrying the FGFR3^{S249C} mutation.

97-7 (FGFR3^{S249C}) cells have not been demonstrated to produce tumors in subcutaneous xenografts in nude mice, which may be due to the dependence of tumor cells on tissue-specific microenvironmental factors (12). However, the current study was not able to verify the effects of the FGFR3^{S249C} mutation on BCa chemosensitivity *in vivo*. It is therefore important to develop a FGFR3 mutant BCa *in vivo* model and in particular, an *in-situ* model, for studying the effects of the FGFR3 mutation on chemoresistance and determining associated underlying mechanisms.

In summary, the *in vitro* analysis used in the current study demonstrated that the FGFR3^{S249C} mutation in BCa cells promoted chemoresistance, at least in part, via the activation of the Akt signaling pathway. The results also indicated that the FGFR3^{S249C} mutation may be developed as a biomarker for the detection of chemosensitivity and that targeting Akt signaling may be a promising treatment strategy for improving chemosensitivity in patients with BCa carrying the FGFR3^{S249C} mutation.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

XX, JL and AT designed the current study. XX, YZ and MF performed the experiments. XX analyzed the data and XX and JL wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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